

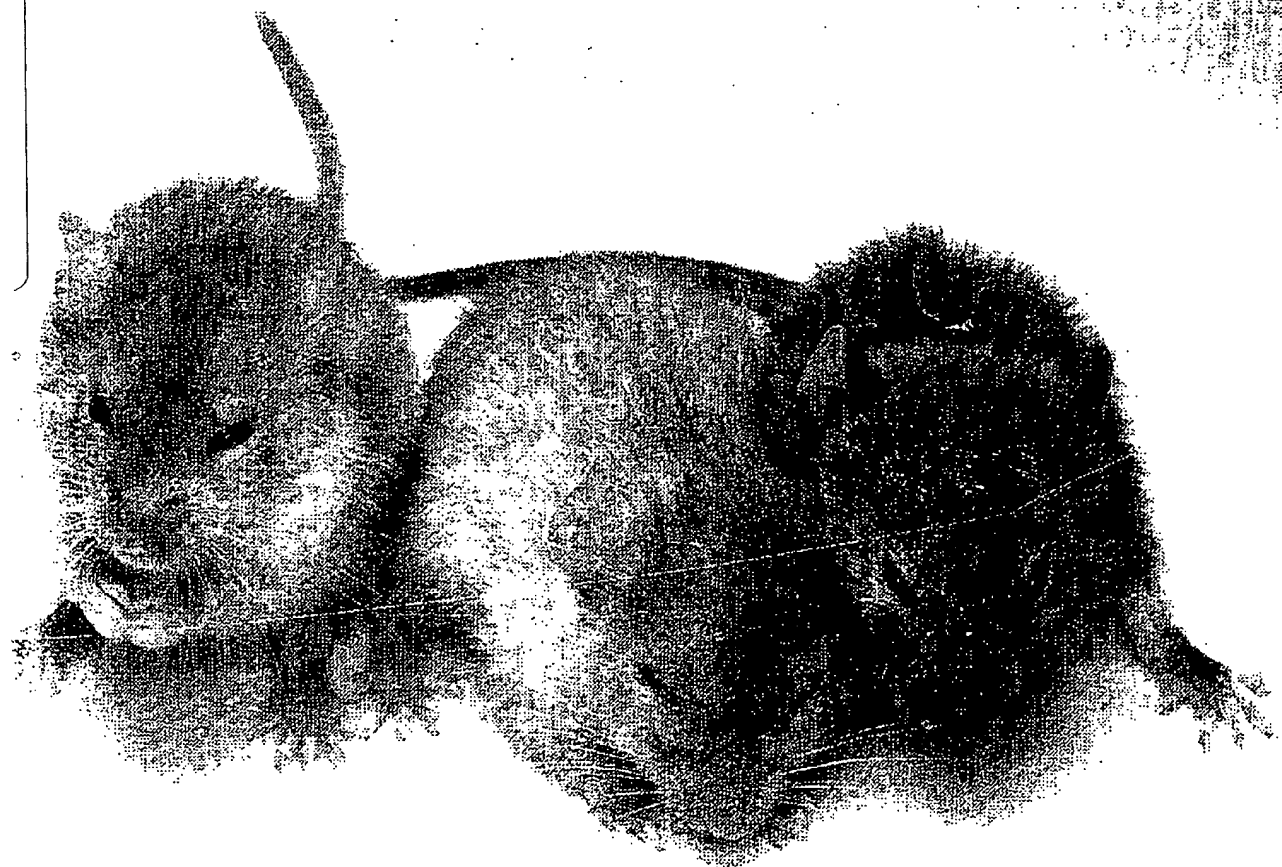
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Appendix O

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SV40-Transformed Simian Cells Support the Replication of Early SV40 Mutants

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Summary

CV-1, an established line of simian cells permissive for lytic growth of SV40, were transformed by an origin-defective mutant of SV40 which codes for wild-type T antigen. Three transformed lines (COS-1, -3, -7) were established and found to contain T antigen; retain complete permissiveness for lytic growth of SV40; support the replication of tsA209 virus at 40°C; and support the replication of pure populations of SV40 mutants with deletions in the early region. One of the lines (COS-1) contains a single integrated copy of the complete early region of SV40 DNA. These cells are possible hosts for the propagation of pure populations of recombinant SV40 viruses.

Introduction

Some of the virus-specific proteins expressed in cells transformed by DNA tumor viruses are responsible for maintenance of the transformed phenotype (Tooze, 1980). Furthermore, viral proteins synthesized in transformed permissive cells can be utilized to complement the growth of superinfecting mutant viruses. For example, permissive cell lines transformed by adenoviruses (Shiroki and Shimojo, 1971; Graham et al., 1977; Grodzicker and Klessig, 1980) and herpes simplex virus (Kimura et al., 1974; Macnab and Timbury, 1976) have been isolated. Using such cells as permissive hosts, host-range mutants of Ad5 (Harrison et al., 1977), deletion mutants of Ad5 (Jones and Shenk, 1979), ts mutants of Ad12 (Shiroki et al., 1976) and ts mutants of herpes simplex virus type 2 (Kimura et al., 1974; Macnab and Timbury, 1976) were successfully propagated because of complementation by viral proteins specified by the integrated viral sequences. However, attempts to develop a similar system for papovaviruses have not been as successful. For example, simian cells transformed by SV40 are either resistant to superinfection (Shiroki and Shimojo, 1971; Gluzman, unpublished data) or are unable to complement the growth of defective viruses (Gluzman et al., 1977a). More encouraging results have been obtained with polyoma virus; host-range mutants have been isolated that grow better on polyoma-transformed mouse cells than on their untransformed counterparts (Benjamin, 1970). However, the enhanced growth of these mutants was not due to direct complementation by viral products expressed in the polyoma-transformed mouse cells (Goldman and Benjamin, 1975).

This paper describes the development and properties of a new line of simian cells, obtained by transformation of CV-1 cells with an origin-defective mutant of SV40 (Gluzman et al., 1980a, 1980b). These cells are permissive to infection with wild-type SV40. They support replication of the DNA of SV40 tsA mutants at nonpermissive temperature, as well as the growth of pure populations of defective viruses with deletions in the early region.

Results

Transformation of CV-1 Cells by SV40 Origin-Defective Mutant DNA

Transformation was carried out using SV40 DNA cloned in plasmids and propagated in *E. coli*. The plasmid DNAs contain either the origin-defective mutant 6-1 or wild-type. The wild-type SV40 recombinant (wild-type plasmid) DNA consists of a complete SV40 genome inserted into the Bam HI site of the modified vector pMK16 #1. The mutant 6-1 DNA was derived from this wild-type plasmid DNA by deleting six nucleotides at the origin of viral DNA replication (Gluzman et al., 1980a, 1980b). Freshly seeded monolayers of CV-1 or TC7 cells ($\sim 5 \times 10^6$ cells/10 cm plate) were transfected with 6-1 or wild-type plasmid DNA using either the DEAE-dextran or calcium technique and the cultures treated as described in Experimental Procedures. Both CV-1 and TC7 cells transfected with wild-type SV40 plasmid DNA eventually gave rise to infectious virus, and most of the cultures lysed after 6 weeks; the DNA of the released infectious viruses contained either small insertions of pMK16 #1 DNA, originating from the vector-SV40 Bam HI junction, or small deletions of SV40 sequences around the Bam HI site (Y. Gluzman, manuscript in preparation). The morphology of the cells on the few plates of CV-1 or TC7 cells that survived transfection with the wild-type plasmid remained indistinguishable from the mock-infected cultures.

No infectious virus was detected in cells transfected with 6-1 DNA. Transformed colonies were first observed at the end of the fourth week and after one and a half months their number reached 10-20 colonies/plate. Transformed colonies emerged only in cultures of CV-1 cells transfected with 6-1 DNA using the Ca^{++} precipitation technique. Transformed foci, composed of densely growing but flat cells, were isolated and transferred into 2.5 cm plates. The mass cultures, derived from individual colonies, contain varying proportions of T antigen-positive cells. Attempts to subclone T antigen-positive cells from early passages were unsuccessful, and all isolated single colonies were T antigen-negative. However, after passaging the mixed cultures of transformed and normal cells for 1-2 months, more than 98% of the cells were T antigen-positive. Three of these uncloned cell lines were chosen for further work and were designated

COS lines (CV-1 origin, SV40) 1, 3 and 7. Each of these cell lines sheds cells upon reaching confluency but can easily survive for 6–8 days as a monolayer. All three lines were used in this work as uncloned populations. Recently, using COS-1 cells that were passaged for 2 months, single colonies have been purified from either soft agar (cloning efficiency 1%) or from liquid medium (cloning efficiency 10%) and found to be similar to the parental transformed cells.

Structure of SV40 DNA in the COS-1 Line

The structure of the SV40 DNA in the COS-1 line was determined by the Southern transfer technique (Southern, 1975). High molecular weight DNA was isolated from either transformed COS-1 or the parental CV-1 cell lines. DNA of the 6-1 mutant plasmid was added to DNA from CV-1 cells in the weight ratio 2:1 $\times 10^6$ (equivalent to 1 copy per cell) and this mixture is referred to in all experiments as a "reconstruction DNA." The DNAs were digested with a variety of restriction enzymes, fractionated on agarose gels and transferred to nitrocellulose filters. Viral and vector sequences were detected by hybridization with ^{32}P -labeled DNAs and visualized by autoradiography (Figure 1). Digestion of COS-1 DNA with either Bgl I or Bgl II, enzymes which do not cut the parental 6-1 DNA, produced two bands, which are different for each enzyme. Bands comigrating with supercoiled or nicked circular forms of the plasmid 6-1 were absent,

suggesting that the viral DNA in COS-1 cells is present only in integrated form. The parental 6-1 plasmid DNA contains two Bam HI sites, so that digestion of reconstruction DNA with Bam HI produces two bands: linear SV40 and linear plasmid DNA (Figure 1a). However, digestion of COS-1 DNA with Bam HI did not yield either of these fragments, suggesting that neither of the insertions contains both Bam HI sites (Figure 1a). The COS-1 DNA contains two insertions of 6-1 plasmid DNA, which complicates the determination of the structure of each single insert. Therefore two different types of analysis were undertaken: screening for the presence of Bam HI, Eco RI and Kpn I restriction sites in each insert; and hybridization with ^{32}P -DNA probes specific for particular regions of 6-1 plasmid. The Bgl II digests contain two fragments about 12 kb (insert A) and 4.8 kb (insert B) in length, which are easily resolved on agarose gel (Figures 1a and 1b). Therefore all further analysis of the structure of the integrated DNA was performed on DNA, predigested with Bgl II; all digests shown in Figures 1b, 1c and 1d are double digests of Bgl II and the indicated enzymes. The results of digestions with Bam HI, Eco RI and Kpn I are shown in Figure 1b. All experiments were done in parallel on COS-1 (1) and reconstruction (R) DNAs. None of the three digests of COS-1 DNA contained the fragments produced by digestion of reconstruction DNA. The apparent comigration of the small Bam HI fragment and linear pMK16 #1 DNA is a coincidence

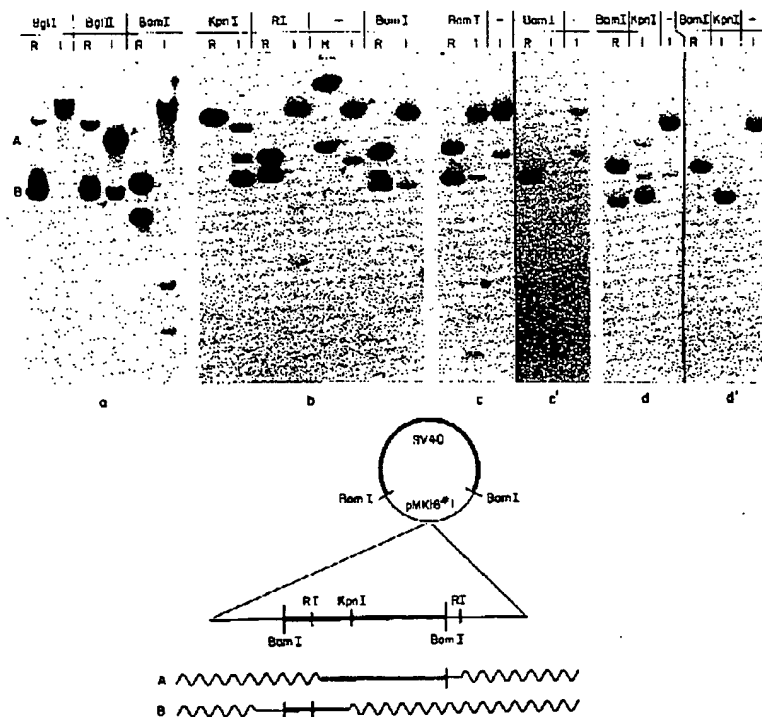


Figure 1. Organization of SV40 DNA in COS-1 Cell Line

(Top) Detection of restriction fragments from COS-1 cells containing SV40 and pMK16 #1 DNAs. High molecular weight DNA from COS-1 cells (1) or reconstruction DNA (R) was digested with different restriction enzymes; fragments were separated by electrophoresis on agarose gels, transferred to nitrocellulose filters and hybridized with radioactive DNAs, specific for particular parts of the 6-1 recombinant plasmid. (a) 0.8% Agarose gel, hybridization probe total 6-1 DNA. All DNA samples shown in (b), (c) and (d) were predigested with Bgl II enzyme and separated on a 1% agarose gel. (b) Hybridization probe total 6-1 DNA. (c) and (c'). The same blot was originally hybridized to ^{32}P -pMK16 #1 probe (c') and after exposure was rehybridized with total ^{32}P 6-1 DNA (c). (d) and (d') Two identical gels were hybridized with ^{32}P -Bgl I/Bam HI fragment of SV40 which encodes the T antigen (d') and total ^{32}P -6-1 DNA (d).

(Bottom) Physical map of the two inserts of viral DNA in COS-1 cells. Circular 6-1 DNA, which consists of viral (thick line) and pMK16 #1 (thin line) genomes, was opened at the middle of pMK16 #1 sequences in order to align it with the linear map of the integrated genome. Wavy line: cellular sequences; broken lines: approximate border of viral or vector sequences with cellular DNA. Inserts A and B, produced by Bgl II digestion, are indicated by arrows on the autoradiograms in (a) and (b).

and results shown in Figure 1c demonstrate that they are unrelated fragments. The larger Bgl II fragment (insert A) is sensitive to Kpn I and Bam HI but not to Eco RI. The smaller Bgl II fragment (insert B) is sensitive to Eco RI and Bam HI but not to Kpn I. The above enzymes all cut one or both inserts within sequences of 6-1 plasmid DNA because digestion with these enzymes increases the number of fragments that hybridize to total 6-1 DNA.

Hybridizations of different digests with ^{32}P -DNA probes, specific for particular regions of the 6-1 plasmid, allowed the estimation of the amount and location of pMK16 #1 DNA in both the A and B inserts (Figures 1c and 1d), and the determination of the regions of SV40 DNA present in each insert, (Figures 1d and 1d'). The amount and the location of the vector DNA in the inserts were determined by hybridization with ^{32}P -pMK16 #1 DNA (Figure 1c'). The same blot was rehybridized with total 6-1 DNA probe (Figure 1c). The amount of vector DNA in the COS-1 cells was estimated by comparing the intensity of the hybridization with vector probe to the reconstruction and COS-1 DNAs. Both inserts contain only small amounts of pMK16 #1 DNA and each insert has only one of the two Bam HI sites between the vector and SV40 DNA. To determine whether the inserts contain overlapping parts of SV40 DNA, two identical blots were hybridized with either a fragment of SV40 DNA encoding T antigen (Bgl I - Bam HI 0.66 - 0.15 map units) or total 6-1 probe (respectively, Figure 1d' and Figure 1d). The result shows that only insert A contains the sequence encoding T antigen.

The structure of the deduced integrated plasmid DNA in COS-1 cells is diagrammed in Figure 1 Bottom. It shows the presence of two inserts of the plasmid 6-1 DNA in these cells. Insert A contains information encoding T antigen, while insert B contains SV40 sequences partially encoding late viral proteins. Both inserts contain only small parts of the vector DNA.

Replication of Mutant SV40 tsA209 In Transformed Cells at the Nonpermissive Temperature

The SV40 mutant tsA209 is incapable of replicating its DNA at 40°C (Chou et al., 1974). The ability of the COS lines to support the replication of this mutant under restrictive conditions was determined by measuring both viral DNA replication and growth at 40°C. Viral DNA replication in different permissive cells was assayed by incorporation of ^{32}P into newly synthesized viral DNA between 30 and 34 hours after infection (Figure 2). Both supercoiled (Component I) and nicked SV40 circles (Component II) were resolved on 1.4% agarose gels and visualized by autoradiography. Three COS lines (1, 3 and 7), the parental CV-1 cell line and C2 cells (CV-1 cells transformed by ultraviolet-irradiated SV40; properties of C2 cells are described in the fusion experiment (see below) and in the Discussion) were tested for their ability to replicate

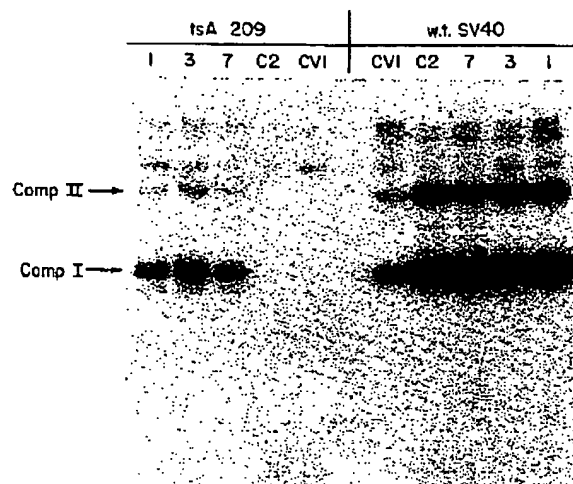


Figure 2. Replication of Wild-Type and tsA209 SV40 DNA in Normal CV-1, Transformed COS-1, -3, -7 and C2 Cells at 40°C

Fresh confluent monolayers of these cells (2.5 cm Petri dish) were infected with 10^7 pfu/plate of either wild-type (w.t.) or tsA209 mutant viruses at 40°C. Thirty hours later inorganic ^{32}P was added at 40°C, and 4 hr later viral DNA was collected by the Hirt procedure (Hirt, 1967) and purified and 40% of the total material was fractionated on a 1.2% agarose gel. The gel was dried and exposed on XR-1 Kodak film for 3 hr.

either wild-type or tsA 209 DNAs at 40°C. All cell lines were permissive for wild-type SV40 DNA replication. A smaller amount of ^{32}P was incorporated into wild-type SV40 DNA extracted from CV-1 cells than from the transformed cells, because there are fewer CV-1 cells per plate. Infection with SV40 tsA209 resulted in viral DNA replication only in the COS lines. No incorporation of ^{32}P into viral DNA was detected in either C2 or CV-1 cells even after the same gel was exposed 5 times longer.

The ability of the transformed lines to complement the replication of SV40 tsA209 at 40°C was also evaluated by determining the virus yield following a single cycle of infection at low multiplicity of infection (MOI). Cells monolayers were infected at 40°C with wild-type or mutant virus at an MOI of 10^{-4} – 10^{-3} pfu/cell. At different times, plates were frozen, infected cells were collected and the virus yield was determined by plaque assay at 33°C (Figure 3). Assuming that in all cases the same proportion of virions initiated the infection and since only a small proportion of cells (0.01–0.1%) was infected, it is possible to interpret the virus yield per plate (Figure 3) as a direct reflection of virus yield per cell. This experiment shows that after infection at 40°C the wild-type virus grows on CV-1 cells and all three transformed lines, but the tsA209 mutant grows only on the COS lines. The rate and extent of tsA209 growth is comparable to the growth of wild-type virus on the transformed lines. These results are in good agreement with the DNA replication experiment described above (Figure 2).

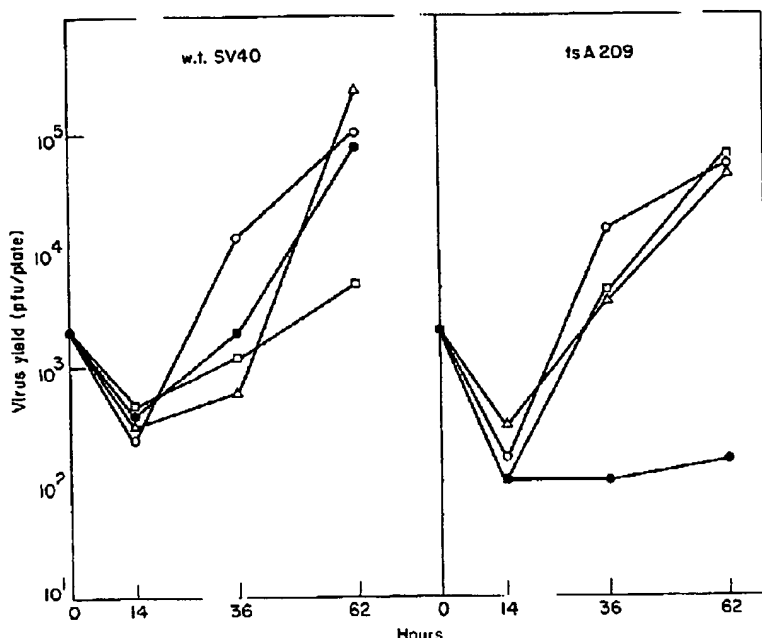


Figure 3. Growth of Wild-Type and tsA209 Viruses on Normal CV-1 and Transformed COS-1, -3, -7 Cells.

Monolayers (10^6 – 10^7 cells/plate) of COS-1, -3, -7 and CV-1 cells were infected with 2×10^5 pfu/plate of either wild-type (w.t.) or tsA209 viruses at 40°C . At each time point, the total virus yield was harvested by freeze-thawing and sonicating the cultures. The virus titer of either virus was determined by plaque assay on CV-1 cells at 33°C , and multiplied by 5 (total volume is 5 ml/plate) to give the virus yield/plate (●—● CV-1; (○—○) COS-1; (□—□) COS-3; (Δ—Δ) COS-7.

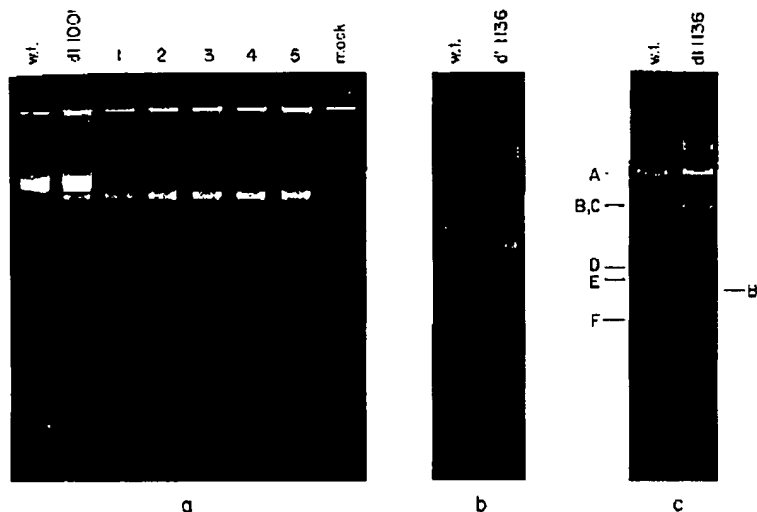
SV40 Mutants with Deletions in the Early Region Can Replicate in COS-1 Cells in the Absence of Helper Virus

The ability of the COS lines to support the replication of a tsA mutant at the nonpermissive temperature suggests that these cells may be used as hosts for the replication of non-conditionally lethal early mutants. Two such viruses were tested: the early SV40 deletion mutant dl 1001 (Lai and Nathans, 1974); and the deletion mutant dl 1136, which is missing 13% of the SV40 genome between 0.47 to 0.60 map units and which was propagated in a plasmid in *E. coli* (J. Pipas, personal communication).

The stock of dl 1001 virus contained a helper virus, an SV40 tsB mutant. Separation of these two viruses was achieved by infecting COS-1 cells in the miniwells with virus at high dilution. This procedure was used instead of conventional plaque purification because COS cells do not survive under an agar layer for the period of time required for plaque development. After two consecutive passages of the appropriate dilutions at 40°C , cytopathic effect (CPE) developed in some miniwells containing COS-1 but not in parallel dilutions in miniwells containing CV-1 cells. Five virus lysates from separate miniwells of COS-1 cells were collected and used to infect fresh monolayers of COS-1 cells. Fifty hours after infection, viral DNA was collected by the Hirt procedure, purified and analyzed on a 1.4% agarose gel (Figure 4a). The parental virus lysate which caused CPE on CV-1 cells was used in this experiment as a control. Two distinct bands are present in the control DNA: a larger DNA (tsB4) which is equal in size to the wild type marker, and a shorter DNA which is dl 1001. All five lysates derived from

growth on COS-1 cells yielded a homogeneous DNA band which comigrated with dl 1001 DNA. Digestion of the viral DNA from two of these samples (2 and 3) with Hind III produced fragments identical to those obtained by digesting the dl 1001 DNA with this enzyme (data not shown).

The second early SV40 deletion mutant (dl 1136) was grown on COS-1 cells using the DEAE-dextran procedure. The DNA of dl 1136 has been inserted into the Bam HI site of pBR322 and propagated as a recombinant plasmid (J. Pipas, personal communication). Total plasmid DNA was digested with Bam HI and Tha I (Tha I cleaves pBR322 but not SV40 DNA). Linear dl 1136 DNA was purified by gel electrophoresis, extracted from the gel, ligated and used to transfect COS-1 cells at 37°C . After 3 days infected cells were harvested and the resulting virus was used to reinfect fresh monolayers of COS-1 cells, which lysed 10 days later. Infection of COS-1 cells with the viral lysate resulted in replication of homogeneous viral DNA that was shorter in size than the wild-type SV40 marker (Figure 4b). The viral DNA sample shown was contaminated with cellular DNA; so the viral DNA was partially purified by gel electrophoresis before digestion with Hind III (Figure 4c). The Hind III pattern of dl 1136 differs from wild-type SV40 by only one fragment; the SV40 B fragment (0.425 → 0.65 map units) is missing in dl 1136 and a new fragment (B'), which is about 700 bp shorter, is found. These results clearly show that pure populations of early deletion mutants can be propagated in COS lines without any helper virus. The titer of mutants in the final COS lysate, estimated by its ability to induce SV40-specific V antigen in COS cells, is 10^6 – 10^7 pfu/ml.



(a) Hind III digest of wild-type (w.t.) and dl 1136 DNAs (b). DNA of dl 1136 mutant was contaminated with cellular DNA, so it was partially purified on agarose gel before digestion (DNA zone above fragment A in dl 1136 slot is due to a residual amount of cellular DNA).

Figure 4. Replication of Early Deletion Mutants of SV40 on COS-1 Cells

COS-1 cells were infected with different viral lysates. Fifty hours later viral DNA was collected by the Hirt procedure, purified and separated by electrophoresis on a 1.4% agarose gel (a, b) or digested with Hind III enzyme before electrophoresis (c). DNA fragments were visualized and photographed after staining in ethidium bromide.

(a) Mixed population of dl 1001 and tsB4 mutant was used as a control lysate (marked dl 1001). Five independent viral lysates obtained from an end-point dilution infection of COS-1 cells with dl 1001 were used to reinfect COS-1 cells for preparation of viral DNA (1-5).

(b) Cloned DNA of dl 1136 mutants was used to transfect COS-1 cells by the DEAE-dextran technique. The resulting viral lysate (dl 1136) was used to reinfect COS-1 cells for preparation of viral DNA.

The mutant dl 1136 DNA initially used to infect COS-1 cells had been cloned in *E. coli* and was therefore free of any contaminating wild-type viral DNA. In spite of this, wild-type virus could, in some instances, be detected in very small quantities (10-500 pfu/ml) after one or two passages of dl 1136 on COS-1 cells. This phenomenon occurred with low frequency and sometimes wild-type virus was detectable after only 2-3 sequential passages. The most plausible explanation is that a recombination event between superinfecting dl 1136 and the integrated viral genome has occurred to generate wild-type virus.

Fusion of COS Lines with C6 Cells Results in Excision and Replication of Integrated SV40 DNA from C6 Cells

C6 cells, like C2, are incapable of supporting the growth of tsA mutants at 40°C. They contain SV40 DNA only in an integrated form, even though the cells are permissive for the growth of superinfecting wild-type SV40. The T antigen(s) synthesized in C2 or C6 cells is competent for maintenance of the transformed phenotype but is unable to initiate viral DNA replication (Gluzman et al., 1977a).

To determine whether the T antigen from the COS lines could induce viral DNA replication in C6 cells, equal numbers of C6, COS (1, 3 and 7) and CV-1 cells were mixed in all possible pairwise combinations, plated out together and treated with polyethylene glycol 24 hours later to induce cell fusion. Three days after treatment low molecular weight DNA was purified, digested with Bgl I and analyzed by Southern (1975) hybridization using a ³²P-labeled SV40 probe. The results are shown in Figure 5. No viral DNA replication was observed after fusion of CV-1 cells with C6 or COS-3. However fusion of C6 cells with

any of the COS lines resulted in efficient replication of a very heterogeneous population of molecules containing SV40 sequences (see Discussion).

Discussion

To establish a host cell line suitable for the propagation of pure populations of either early host-range mutants of SV40 or recombinant SV40 viruses, with foreign DNA substituting for viral early region, two requirements must be met: T antigen expressed in this line must possess all the properties which are required for synthesis of viral DNA; and the cells have to retain susceptibility to superinfection with SV40. Efficient viral DNA replication requires a viral origin, functional T antigen (first requirement) and host permissiveness (second requirement). If the transformation of permissive cells and efficient viral DNA replication are incompatible events, then the elimination of the viral origin is the simplest way to affect the replication capability of viral DNA and to preserve these requirements. We have recently isolated and characterized a group of origin-defective mutants (Gluzman et al., 1980a, 1980b). They have suffered deletions around the Bgl I site and most of them produce wild-type T antigen; all T antigen producers transform Rat-1 cells efficiently. One such mutant, 6-1, which produces functional T antigen, has lost six nucleotides around the Bgl I site. After infection of CV-1 cells with supercoiled 6-1 DNA transformed colonies were obtained, although the efficiency of transformation (20-30 colonies/1 µg of DNA/10⁶ cells) was about 10 fold lower than transforming efficiency of 6-1 DNA on Rat-1 cells (Gluzman et al., 1980a). Transformed colonies began to appear after 4-5 weeks and were isolated after 6 weeks or later. These original isolates did not multiply

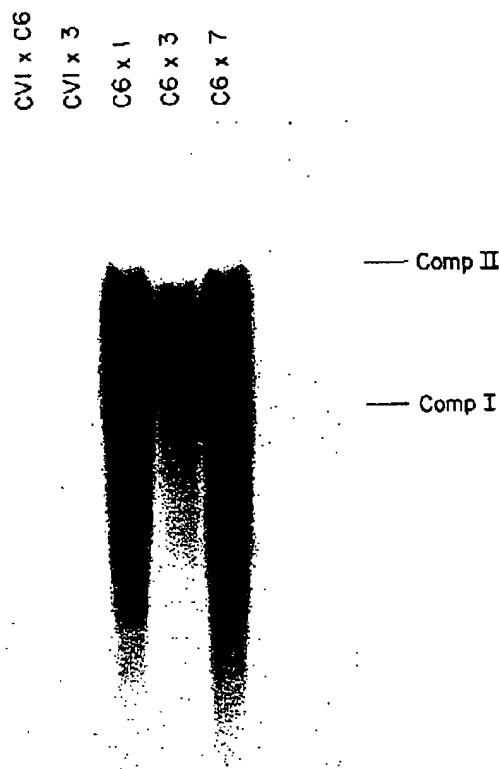


Figure 5. Replication of Viral DNA after Fusion of C6 Cells with COS-1, -3, -7 Cells

Different cell lines were fused using the polyethylene glycol method. Three days later low molecular DNA was extracted by the Hirt procedure, purified, linearized with Bgl I (to facilitate the blotting and to give the size distribution of excised DNAs), separated on a 1.4% agarose gel and transferred to a nitrocellulose filter. SV40 DNA was visualized by hybridization with SV40 ³²P- DNA and exposure to Kodak XR film.

faster than the progenital CV-1 cells. After an additional 1-2 months in culture, however, the transformants outgrew the CV-1 cells, which were present in the original isolates.

Three established lines (COS-1, -3, -7) of transformed cells were developed and more than 98% of the cells in each population were T antigen-positive. All three lines were completely permissive for superinfection with wild-type and tsA mutant virus. The cells grew to high density but only as a monolayer. One of the lines (COS-1) was subcloned in soft agar and in liquid medium; the subclones had the growth properties and complementing activity of the parental line. The reasons for a complicated pathway leading to stable transformation of COS cells are not clear, especially since nonpermissive rat cells, transformed by the same or other origin-defective mutants, expressed the stable transformed phenotype within two weeks of infection (Gluzman et al., 1980a). One might speculate that there are cellular sequences in the monkey

cells identical or similar to the SV40 origin, and functional SV40 T antigen might recognize these sequences, which will initiate an aberrant cellular DNA replication, resulting in an unstable phenotype. Simian (T. McCutchan, M. Singer, personal communication) and human (M. Botchan, personal communication) sequences which have extensive homology to SV40 origin were recently isolated; however, the ability of these regions to respond to SV40 T antigen is not yet established.

Free viral DNA was not detected in COS-1 cells. There are two integrated inserts of parental transforming DNA (6-1); only one of them contains information encoding T antigen. Approximate boundaries of the inserts are depicted in Figure 1. It is interesting to note that the junction between SV40 and cellular sequences occurs in the same region (between map position 0.72 and 1.0) in both inserts. If both of these inserts originated independently from two different parental molecules, then it might suggest that this is a "preferable" region for recombination. Alternatively these two inserts may be the result of a postintegrational break of a single integrated SV40 DNA molecule. To distinguish between these two possibilities it will be necessary to clone the integrated SV40 and define more precisely the cell-virus DNA boundaries.

The most important property of COS lines is their ability to support the growth of early mutants under conditions where they fail to grow on CV-1 cells. This property makes these cells very useful for the propagation of homogeneous populations of early mutants. Furthermore, SV40 has been widely used as a cloning vector for the propagation of procaryotic or eucaryotic DNAs (reviewed in Hamer, 1980), but those recombinant viruses were always propagated together with a helper (usually ts) virus. The development of COS lines removes the need for helper viruses, since the cells should allow the propagation of SV40 with foreign DNA recombined in the early region.

Passaging of early deletion mutants in COS-1 cells results in the appearance of wild-type virus. Nothing is known about the mechanism of recombination between resident and superinfecting virus. The frequency of this event is low and difficult to quantitate; however, low frequency recombination does not prevent the propagation of relatively pure populations of mutants.

Because CV-1 cells transformed by ultraviolet-irradiated virus (C2, C6) are unable to support the replication of viral DNA after infection with tsA at 40°C, it was suggested that they express a mutated T antigen, which is competent for maintenance of transformation but not for viral DNA replication (Gluzman et al., 1977a). Direct evidence to support this idea is provided by the fusion experiment (Figure 5). When a functional T antigen from COS cells is provided in trans by fusing COS cells with C6, the SV40 genome resident in C6 cells can be excised and replicated. C6 cells therefore contain SV40 with mutated T antigen

and functional origin of DNA replication. The ability of COS cells to induce excision and replication of SV40 can be exploited for the excision, amplification and isolation of different genes linked to the SV40 origin. For example, the SV40 DNA from C6 cells, which is present as a single copy, has been successfully cloned from the Hirt supernatant (see the fusion experiment) into the Bam HI site of pBR322; the cloning efficiency was very high—1/10,000 colonies (Y. Gluzman, manuscript in preparation).

SV40 DNA from C6 cells replicates as a very heterogeneous population of molecules. A similar population was observed previously when cells of the SV40-transformed rat line 14B were fused with CV-1 cells (Botchan et al., 1978, 1980). The integrated SV40 DNA in 14B cells, like that in C6 cells (Krieg et al., 1980), contains no duplication of viral sequences. This lack of duplication is probably responsible for the heterogeneous pattern of excision.

Experimental Procedures

Cells, Viruses and Enzymes

The established cell line CV-1 was obtained from ATCC and was grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal calf serum. C2 and C6, which are CV-1/SV40-transformed cell lines (Gluzman et al., 1977a), were propagated in the same medium with 5% calf serum. The SV40 mutant tsA209 (Chou et al., 1974) was propagated and titered as described previously (Gluzman et al., 1977a, 1977b). The dl 1001 virus (Lai and Nathans, 1974) was obtained from W. C. Topp.

All restriction endonucleases were purchased from Bethesda Research Laboratories; digestions were performed under conditions suggested by the producer. DNA polymerase I was purchased from Boehringer-Mannheim.

Source of Recombinant DNAs

Wild-type plasmid is a recombinant DNA containing the complete genome of SV40 inserted into the Bam HI site of the modified vector, pMK16 #1. The origin-defective mutant, 6-1, is a derivative of wild-type plasmid and is missing six nucleotides at the Bgl I site of SV40 DNA. The isolation, structure and biological properties of this mutant have been described (Gluzman et al., 1980a, 1980b). Deletion mutant dl 1136 is a mutant of SV40 which is missing about 700 nucleotides ($\sim 0.47 \rightarrow 0.6$ map units) and is propagated as a recombinant DNA with pBR322 (gift from J. Pipas and D. Nathans). The propagation and preparation of recombinant DNAs has been described (Gluzman et al., 1980a), and handling of recombinant DNAs was according to the NIH guidelines.

Transformation of Permissive Cells with SV40 Origin-Defective 6-1 DNA

Fresh monolayers of CV1 or TC7 cells ($\sim 5 \times 10^6$ cells/10 cm plate) were transfected with either 6-1 or wild-type plasmid DNAs. Transfections were performed using either the DEAE-dextran (McCutchan and Pagano, 1968) or the modified calcium (Wigler et al., 1978) technique. Twenty-four hours later, each culture was divided into six subcultures and incubated for 6 weeks; medium was changed every 7–10 days. Transformed foci were isolated with Pasteur pipettes and transferred into 2.5 cm Petri dishes. After passaging for 1–2 months these established cell cultures, named COS-1, -3 and -7, were used for further work.

Determination of the Structure of the Integrated SV40 DNA in COS-1 Cell Lines

High molecular weight DNA was isolated from either transformed COS-1 or the parental CV-1 cells. DNA of the 6-1 mutant plasmid

was added to the high molecular weight DNA from CV-1 cells in the weight ratio 2:1 $\times 10^6$ and this mixture was used as reconstruction DNA. The DNAs were digested with a variety of restriction enzymes, fractionated on agarose gels and transferred to nitrocellulose filters (Southern, 1975). Viral and vector sequences were detected by hybridization with ^{32}P -labeled nick-translated probes (Rigby et al., 1977) consisting of either whole 6-1 plasmid or specific regions of this DNA.

Viral DNA Synthesis

CV-1, C2 and COS-1, -3, -7 cell lines were plated into 2.5 cm Petri dishes and 24 hr later were infected with wild-type or tsA209 viruses (10^7 pfu/plate) at 40°C. Thirty hours after infection medium was replaced with 1.5 ml prewarmed solution containing 150 μCi of inorganic ^{32}P (NEN), 2% FBS and phosphate-free medium. After labeling for 4 hr at 40°C viral DNA was extracted by the Hirt procedure, treated with RNAase (5 $\mu\text{g}/\text{ml}$ RNAase at 37°C for 1 hr) and precipitated with ethanol. The pellet was resuspended and 40% of the total material was electrophoresed on a 1.2% agarose gel. The dried gel was exposed for 3 and 15 hr on XR-1 film (Kodak).

Rescue of SV40 DNA from Transformed C6 Cells by Fusion with COS Cell Lines

Cellular fusion was accomplished by polyethylene glycol treatment (Gluzman et al., 1980a). Equal numbers of cells (2×10^6 cells) were mixed and plated on Petri dishes. Seventy-two hours later, low molecular weight DNA was isolated and purified as described under Viral DNA Synthesis. Purified DNA was linearized with Bgl I (to facilitate the transfer and to estimate the size of excised molecules), separated on 1.4% agarose gel, transferred to nitrocellulose filters (Southern, 1975) and hybridized to the ^{32}P -labeled SV40 nick-translated probe (Rigby et al., 1977).

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